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TITLE: The Prevalence and Importance of Epithelial Plasticity in Metastatic Prostate Cancer

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14. ABSTRACT

Metastasis is the leading cause of prostate cancer-associated death. The metastatic cascade is thought to be mediated by phenotypic plasticity, which includes epithelial-mesenchymal transitions and mesenchymal-epithelial transitions (EMT and MET, respectively) in solid tumors. There is a substantial amount of data to support the role of EMT in seeding metastasic sites in prostate cancer, however, less is known about the importance of MET in the metastatic cascade. Previous studies suggest that MET enables the growth of macrometastases outside of the prostate, but whether or not MET is required for metastatic colonization has not yet been addressed. To determine the frequency of MET and, most importantly, whether MET is required for prostate cancer metastasis in vivo, we created novel alternative splicing reporters. Interestingly, we found that in two models of prostate cancer that MET are rare and that prostate cancer metastasis can occur independently of MET. Thus, we conclude that undergoing MET is not a prerequisite of prostate cancer metastasis.

15. SUBJECT TERMS

Prostate cancer, phenotypic plasticity, mesenchymal-epithelial transitions, migration, invasion, lineage tracing, metastasis

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Introduction

Metastasis requires cancer cells to adapt to their microenvironment, which includes their ability to invade surrounding tissues and proliferate. Epithelial carcinoma cells are non-invasive and proliferative, allowing them to grow locally whereas cells that have transitioned to a mesenchymal state are invasive and less proliferative. Cells that have undergone this epithelial-mesenchymal transition (EMT) are adept at degrading the basement membrane and entering the circulatory system, which are the first steps of metastasis.

Substantial evidence supports the role of EMT in seeding metastases, however, less is known about the importance of the reverse transition, mesenchymal-epithelial transitions (MET). It has been suggested that transitioning back to an epithelial, and thus proliferative, state for the outgrowth of a micrometastatic lesion. The goal of this study is to determine the frequency of MET during prostate cancer (PC) progression and whether transitioning to an epithelial state is required for the formation of PC metastases. To this end, we have used a synthetic biology approach to develop novel alternative splicing reporters that enabled us to observe the rate of MET and determine whether cells that are unable to undergo MET are capable of forming macrometastases in rodent models of PC. Our results indicate that, at least in the rat and human prostate adenocarcinoma models that we used, MET are rare during tumorigenesis and metastasis and that metastasis can occur independently of MET. Future directions are aimed at determining whether the PC cells that have undergone MET *in vivo* are as tumorigenic and metastatic as those that remain mesenchymal.

Keywords

Overall Project Summary

Current Objectives

The hypothesis of the proposed research is that MET occurs frequently during PC progression and is important in metastatic colonization. The current objectives of this study are to: (i) Determine the frequency of MET using rodent models of PC, (ii) Test the importance of MET in PC tumorigenesis and metastasis *in vivo*, and (iii) Examine the tumorigenicity and metastatic potential of PC cells that have previously undergone MET *in vivo*.

Results, progress, and accomplishments

The first and second objectives of this proposal have largely been accomplished and culminated in a manuscript that will be submitted in November. References to Figures from this manuscript are indicated throughout (see also the Appendix). Our first objective was to determine the frequency of MET in rodent models of PC. To do this, we used a synthetic biology approach to generate two constructs that could permanently mark a cell that had undergone MET. The rationale for this approach relied on previous findings from our laboratory that indicate that alternative splicing of fibroblast growth factor receptor 2 (FGFR2) faithfully reports on epithelial and mesenchymal cell states (*1, 2*). Skipping of exon IIIc is a hallmark of epithelial cells, whereas inclusion marks mesenchymal cells. Therefore, we exploited the cell-type specific alternative splicing of FGFR2 to create the ^{Ecad}CreIIIcI^2 plasmid, which contains mesenchymal exon IIIc and the flanking intronic regions of FGFR2 interrupted by the Cre open reading frame (ORF). To provide increased cell type specificity, the ORF is under the control of the E-cadherin promoter. Therefore, activation of Cre requires epithelial-specific activation of the E-cadherin promoter and skipping of exon IIIc. To mark cells that had undergone MET, we also used the RG construct that consists of DsRed flanked by loxP sites coupled to EGFP whose expression is driven by the CMV promoter (Figure 2A). To prevent co-expression of DsRed and EGFP, both ORFs contain stop codons. Prior to using these reporters *in vivo*, they were validated in epithelial and mesenchymal rat PC cell lines *in vitro*. Briefly, epithelial DT cells harboring the plasmids express Cre, which results in EGFP expression, whereas mesenchymal AT3 cells lack Cre and thus express DsRed (See Figure 2B in the Preliminary Studies section of the original proposal).

To determine the frequency of MET in primary tumors, mesenchymal AT3 cells harboring the ^{Ecad}CreIIIcI^2 and RG reporters were injected subcutaneously (s.c.) into the flanks of male syngeneic Copenhagen or nude rats, and primary tumors were collected eight days later for analyses. As observed with AT3 cells *in vitro*, tumors from cells harboring ^{Ecad}CreIIIcI^2 and RG were predominantly DsRed+, but contained multiple foci of EGFP+ cells (Figure 3A; white arrows). To determine the frequency of MET in metastases, AT3 cells with ^{Ecad}CreIIIcI^2 and RG were injected via the tail vein (t.v.), and lungs were harvested after eight days[†]. We observed EGFP+ AT3 cells only rarely within lung macrometastases (Figures 3C and 3D) suggesting that the majority of these metastases formed from cells that had never undergone MET. The mesenchymal cell state of the primary tumors and metastases was also verified by staining tumor sections with epithelial- and mesenchymal-specific biomarkers (Figure 3B and E). The results from the reporters and staining indicate that MET is a rare event during both AT3 tumorigenesis and formation of metastatic colonies in the lungs.

While the ^{Ecad}CreIIIcI^2 reporter can inform on the frequency of MET, it does not address the importance of MET in the ontogeny of PC tumorigenesis and metastasis. To achieve this, we used the ^{Ecad}DipIIIcI^2 plasmid, which specifically kills cells that have undergone MET. The ^{Ecad}DipIIcI^2 reporter is the same as ^{Ecad}CreIIIcI^2 except the Cre ORF is replaced with the diphtheria toxin ORF. Prior to using these reporters *in vivo*, they were validated in the epithelial DT and mesenchymal AT3 cell lines *in vitro*. Briefly, DT and AT3 cells harboring the ^{Ecad}DipIIIcI^2 plasmid, which contains a blasticidin resistance gene, were monitored for growth. After six days of blasticidin selection, only AT3 cells harboring ^{Ecad}DipIIIcI^2 survived treatment with blasticidin (Figure 4A).

To determine whether MET was required for metastases, AT3 cells harboring ^{Ecad}DipIIIcI^2 were injected into nude rats via the t.v. To visualize the AT3 cells *in vivo*, regardless of their cell state, cells also harbored a plasmid that constitutively expressed EGFP (Gint). AT3-Gint cells that harbored a control non-suicide splicing reporter, RIIIcI^2, were also injected into nude rats to mark cells that have undergone MET. The RIIIcI^2 reporter is similar to ^{Ecad}DipIIIcI^2 except that it is driven by the CMV promoter and exon IIIc interrupts the DsRed ORF. Cells harboring this construct will be viable, regardless of their cell state, and will be DsRed+ if they have undergone MET. Twelve days post-injection, lungs were harvested and the number of macrometastases were counted. There was no qualitative differences in the size, gross morphology, or number of the metastases between control rats and those injected with ^{Ecad}DipIIIcI^2 cells (Figure 4C and D). Overall, the results from lineage tracing reporters, suicide reporters, and biomarker staining clearly indicate that MET is not required for tumor formation or metastatic colonization of the lungs in the mesenchymal AT3 PC model.

To determine whether PC metastasis can occur independently of MET in a human PC model, DU145 cells harboring the ^{Ecad}DipIIIcI^2 reporter were injected into the t.v. of nude mice[‡]. As a control to visualize MET, DU145 cells harboring the control reporter, ^{Ecad}CreIIIcI^2, were also injected. Consistent with the AT3 model, there was no change in the number of metastases in control mice as compared to those injected with ^{Ecad}DipIIIcI^2 cells (Figure 6A). Additionally, there was no evidence of MET in lung metastases in mice injected with DU145 ^{Ecad}DipIIIcI^2 cells as observed by staining tumor sections with epithelial and mesenchymal biomarkers (Figure 6B).

While MET was not shown to be required in our models of rat and human PC metastasis, our reporters did show that MET was required for metastasis of a human uterine carcinosarcoma cell line, CS-99, *in vivo* (Figure 5). Rather unexpectedly, we noted that AT3 tumors resemble carcinosarcomas in that they have an undifferentiated histology, a mesenchymal-like gene expression profile, and a preference for metastasizing to the lungs (*1, 2*). Despite this similarity, AT3 and CS-99 cells take different routes to metastasis.

[†] In the original proposal, retroperitoneal lymph nodes were also going to be monitored for MET. The lymph nodes were not included in our analysis because we had trouble locating them in a sufficient number of rodents.

[‡] In the original proposal, the human PC cell line PC3 was listed as an additional model in which to study MET. We chose to use the DU145 cell line, however, because there is evidence that DU145 cells undergo MET when metastasizing to the sentinel lymph nodes of the prostate (*3*).

Discussion

During carcinoma progression, EMT is generally accepted as a means by which cancer cells gain invasive capabilities and break free from the confines of the primary tumor (*4, 5*).

Several investigations have also suggested that MET may be important for metastatic colonization subsequent to dissemination from the primary tumor. Like these previous studies, the present work also suggests that CS-99 human carcinosarcoma cells require MET to efficiently metastasize in the lungs. Yet, our data also indicate that other, post-EMT cancers can metastasize through a mechanism that appears to be independent of MET.

The finding that MET appears to be critically important for metastasis of some cancers, while others have no apparent need for this transition is reminiscent of predictions by Brabletz (2012) that two "routes to metastasis" exist. Brabletz postulates that some cancers will be plasticity-dependent while other cancers will metastasize by way of one or more plasticity-independent pathways (*6*). Our data provide evidence for both the MET-dependent and MET-independent routes to metastasis. Yet, it remains unclear which genetic and/or environmental factors drive a given cancer type down either of these pathways. AT3s and CS-99s, for example, both represent models of carcinosarcoma. Despite their similarities, AT3s metastasize efficiently via an MET-independent program while CS-99s seem to use an MET-dependent metastatic cascade.

Unlike the carcinosarcoma models, DU145s are a classical model of aggressive prostate adenocarcinoma. Perhaps unexpectedly, DU145s, like AT3 cells, also metastasize independently of MET. Yet, DU145 cells are capable of transitioning to a more epithelial phenotype over successive rounds of i.v. injection and metastasis to the lungs (*3*). Collectively, our data provide experimental validation of initial predictions that cancers undergo metastatic progression via plasticity-dependent and -independent mechanisms (*6*). Together, our data suggest that the same cancer type, and maybe even cells within a single human tumor, may be capable of metastasizing via different routes. Perhaps most importantly, these findings argue that effective treatment of metastatic disease may require treatments that target different metastatic pathways.

Key research accomplishments
- Combinatorial use of regulatory elements enabled design of enzyme-based constructs that can be used to used to report on the frequency of MET
- Genetically-regulated reporters allowed for tracking of phenotypic plasticity in vivo
- Using the novel reporters that we have developed, we have addressed two of the three objectives of this research proposal. We have shown that MET is a rare occurrence during PC progression and, in at least two models, is not required for metastasis
- We have shown that metastasis of carcinosarcoma is dependent on MET

Conclusion

The results obtained from completing objectives one and two of this proposal indicate that metastasis can occur via MET–dependent and –independent routes. This is particularly important because this is the first time that a MET-independent model of metastasis has been shown. The implications of this research suggest that EMT, rather than MET, is a driver of metastasis. Therefore, we propose a change in the development of PC therapeutics toward agents that directly impact cells that have undergone EMT rather than epithelial carcinoma cells.

In the remaining year of the fellowship, I will fulfill objective three of the original research proposal, which is to examine the tumorigenicity and metastatic potential of PC that have previously undergone MET *in vivo*. To accomplish this, I will inject RIIIcI^2/Gint-containing AT3 and DU145 cells s.c. and into the t.v. of rats and nude mice, respectively. Briefly, the control RIIIcI^2 plasmid will allow for MET to be monitored in real-time, with post-MET cells expressing DsRed, whereas the Gint plasmid will fluorescently label AT3 and DU145 cells regardless of their cell state. I will harvest cells that have undergone MET (DsRed+, EGFP+) from primary tumors and metastases and re-inject into their respective species. To determine the tumorigenicity and metastatic potential of these cells, the number and size of primary tumors and metastases will be measured. As a control, cells that have not undergone MET (DsRed-, EGFP+) will also be harvested and re-injected.

Publications, Abstracts, Presentations

    A. Manuscripts

        1. Lay press – None

        2. Peer-reviewed scientific journals – Somarelli, J.A., **Schaeffer, D.**, Marengo, M.S., Bepler, T., Rouse, D., Ware, K.E., Zhao, Y., Buckley, A.F., Epstein, J.I., Virshup, D.M., and M.A. Garcia-Blanco. 2014. Distinct routes to metastasis: plasticity-dependent and plasticity-independent pathways. *Cancer Cell*. Submitted.

        3. Invited articles – None

        4. Abstracts – None

    B. Presentations

    Schaeffer, D., Somarelli, J.A., Hanna, G., Palmer, G.M., and M.A. Garcia-Blanco. 2014. Cellular migration and invasion uncoupled: Increased migration is not required for EMT or increased metastasis in a prostate cancer model. Gordon Research Conference. Post-transcriptional gene regulation: From mechanisms stems therapy. Newport, Rhode Island.

    Somarelli, J.A., Schaeffer, D., Marengo, M., Bepler, T., Rouse, D., Buckley, A.F., Epstein, J.I., Armstrong, A.J., and M.A. Garcia-Blanco. 2014. Splicing reporters reveal new insights about the metastatic cascade. Gordon Research Conference. Post-transcriptional gene regulation: From mechanisms stems therapy. Newport, Rhode Island.

Inventions, Patents, and Licenses
Nothing to report

Reportable outcomes

      The lineage tracing (^{Ecad}CreIIIcI^2 and RG) as well as the suicide (^{Ecad}DipIIIcI^2) constructs represent a novel technology that can be used to address the frequency and importance, respectively, in other types of cancers. In addition, the use of combinatorial regulatory elements can be further adapted to include more cell-type specific control and can therefore be applied to the design of next generation reporters.

Other acheivements

      This research led to the creation of AT3 and DU145 stable cell lines that contain alternative splicing reporters. The work that has been completed has been used as preliminary data and rationale for an Idea Development Award submitted by a fellow postdoctoral fellow, Jason Somarelli, to the Department of Defense Prostate Cancer Research Program (PCRP).

      The support provided by this fellowship also enabled the study of the phenotypic processes that occur during EMT. Briefly, I discovered two model systems wherein epithelial, or pre-EMT, cell lines were more migratory than their mesenchymal, or post-EMT counterparts. While this finding is supported by other types of epithelial cell migration, including keratinocyte migration during wound healing, this was the first report of pre-EMT cells being more migratory than those that had undergone EMT. Additionally, I also demonstrated that while post-EMT cells are less migratory than their epithelial counterparts, they are more invasive. This phenomenon suggests that, contrary to how EMT has been previously defined, migration and invasion are not always positively correlated in post-EMT cancer models. Lastly, I identified the EGFR signaling axis as being a positive regulator of cell migration in pre-EMT cancer cells. This work culminated in a recent publication in Molecular and Cellular Biology (*7*).

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1   **Distinct routes to metastasis: plasticity-dependent and plasticity-independent pathways**

2

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1   **Running title:** Different pathways to cancer metastasis

2   **Key words:** epithelial plasticity; mesenchymal-epithelial transition; lineage tracing; fibroblast growth

3   factor receptor 2; E-cadherin; carcinosarcoma

4   **Summary**

5   The cascade that culminates in macrometastases is thought to be mediated by phenotypic plasticity,

6   including epithelial-mesenchymal and mesenchymal-epithelial transitions (EMT and MET, respectively).

7   While there is substantial support for the role of EMT, much less is known about the importance of MET

8   in driving metastasis. We created novel reporters to test whether MET is required for metastasis in

9   multiple *in vivo* cancer models. Strikingly, we found evidence for MET-dependent and MET-independent

10   metastatic pathways. We conclude that MET is not a prerequisite for metastatic colonization, and that

11   post-EMT cancers can metastasize efficiently in the absence of MET.

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13   **Highlights**

14   • Combinatorial use of regulatory elements enables design of enzyme-based reporters

15   • Genetically-regulated reporters allow tracking of epithelial plasticity *in vivo*

16   • Lineage tracing reveals rare MET-like events in primary tumors and metastases

17   • Metastasis occurs via MET-dependent and MET-independent pathways

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1 **Introduction**

2   Epithelial plasticity, including epithelial-mesenchymal transitions (EMT) and the reverse,

3 mesenchymal-epithelial transitions (MET), is involved in diverse processes, such as development (Lim

4 and Thiery, 2012), wound healing (Nakamura and Tokura, 2011), fibrosis (Carew et al., 2012), and

5 carcinoma metastasis (Thiery, 2002). In the context of metastasis, there is substantial evidence to

6 suggest that EMT confers upon cancer cells the ability to detach from the primary tumor, degrade the

7 basement membrane, and invade locally or distally (De Craene and Berx, 2013; Thiery, 2002). While

8 much of the evidence for EMT in driving metastatic disease comes from preclinical observations, there

9 are also clinical studies that highlight the EMT program as a driver of carcinoma progression. For

10 example, a number of groups have observed upregulation of EMT biomarkers, including TWIST1, SNAI1,

11 ZEB1, and TGF-β, at the invasive front of human cancers (reviewed in (Bastid, 2012)). Similarly, human

12 circulating tumor cells from patients with metastatic breast (Aktas et al., 2009; Armstrong et al., 2011;

13 Raimondi et al., 2011; Yu et al., 2013) and prostate (Armstrong et al., 2011) cancers have also shown

14 expression of stemness and EMT markers.

15   While EMT is important for invasion and seeding of micrometastases, the reversion to an

16 epithelial phenotype, MET, is postulated to be responsible for the establishment of macrometastatic

17 colonies (Brabletz, 2012; De Craene and Berx, 2013; Thiery, 2002). Observations from metastatic breast

18 cancers indicate that E-cadherin, a widely used epithelial-specific biomarker, is upregulated in

19 metastatic sites compared to primary tumors (reviewed in (Gunasinghe et al., 2012)). In addition, a

20 survey of metastatic tumors from pancreatic cancers showed higher E-cadherin expression and lower

21 levels of the E-cadherin transcriptional repressors, ZEB1 and ZEB2, in the larger metastatic tumors

22 (Kurahara et al., 2012). Preclinical investigations have also suggested that MET may be important for

23 metastasis. In a xenograft mouse model of breast cancer, Ocaña et al. (2012) showed that

24 downregulation of the EMT inducing transcription factors PRRX1 and TWIST1 were necessary for

1 metastasis to the lungs (Ocana et al., 2012). Similarly, in a chemically-induced squamous cell carcinoma

2 model, Tsai et al. (2012) induced EMT using tetracycline-inducible TWIST1, a known E-cadherin

3 repressor and master regulator of EMT (Yang et al., 2004). Primary tumor cells isolated from these mice

4 were re-injected via the tail vein under persistent exposure to tetracycline (constant TWIST1 expression)

5 or with tetracycline removed (temporary TWIST1 expression). Importantly, significantly more

6 macrometastatic colonies were observed in the group from which tetracycline was removed, suggesting

7 that MET was partially responsible for formation of macrometastases in this model (Tsai et al., 2012).

8 While these data provide compelling, mechanistic pre-clinical evidence for the role of MET in promoting

9 metastatic colonization in this model system, it is also noteworthy that significantly more metastatic

10 nodules formed (an average of approximately 50 per mouse) from cells ectopically expressing TWIST1

11 compared to the group of mice injected with cells with no TWIST1 expression (an average of less than 25

12 per mouse) (Tsai et al., 2012). These data suggest that TWIST1-induced EMT may be important for

13 metastasis without the need to undergo MET.

14 While a growing body of literature indicates that epithelial plasticity may drive metastatic

15 progression in several types of carcinoma, there also exist a number of reports that call into question

16 the importance of epithelial plasticity in mediating metastatic dissemination and colonization. In

17 particular, work by McCaffrey et al. (2012) revealed that loss of the polarity protein Par3 induced cell

18 detachment, led to upregulation of MMP9, enhanced invasive potential, and increased lung metastases

19 in a murine breast cancer model. Importantly, although the more invasive Par3 knock down cells lost E-

20 cadherin, the invasive cells retained ZO-1, an epithelial-specific marker, and displayed no consistent

21 changes in the mesenchymal markers N-cadherin or vimentin (McCaffrey et al., 2012). Similarly, analysis

22 of matched primary and metastatic breast cancer biopsies indicated that while 85% of metastases

23 exhibited increased E-cadherin expression when compared to primary tumors, the metastases retained

24 mesenchymal markers fibroblast-specific protein 1 (FSP1) and vimentin (Chao et al., 2012). Analysis of

1   prostate cancer primary tumors and metastases also showed increased E-cadherin; however, the

2   amount of E-cadherin was inversely proportional to size of the metastatic nodule, with larger

3   metastases expressing the least E-cadherin compared to smaller metastases (Chao et al., 2012). Tanaka

4   et al. (2012) reported that the mesenchymal marker N-cadherin was expressed in 67% of metastatic

5   prostate cancer biopsies (Tanaka et al., 2010). These preclinical and clinical data challenge the

6   requirement for complete EMT/MET in mediating metastasis and indicate that the metastatic cascade

7   may involve partial rather than complete transitions between phenotypic states. In addition, while

8   EMT/MET may be involved in mediating metastatic colonization in some well differentiated

9   adenocarcinomas, there are a number of post-EMT and/or mesenchymally-derived cancer types that

10   may possess all of the traits necessary for metastatic dissemination in the absence of frank EMT/MET.

11   Along these lines, Brabletz (2012) postulated that two routes to metastasis may exist: one that is

12   plasticity-dependent and another that is plasticity-independent. Brabletz opines that the requirement

13   for MET during metastasis may be a property of differentiated tumors, while MET-independent

14   metastases may exist as a product of genetic alterations that render the need for MET obsolete.

15   Previously, our group developed fluorescence-based reporters of epithelial plasticity to visualize

16   phenotypic transitions *in vivo* (Oltean et al., 2008; Oltean et al., 2006; Somarelli et al., 2013). We

17   reasoned that our MET reporters could be extremely useful to test the hypothesis that MET is a

18   modulator of metastatic colonization. Along these lines, we generated a lineage tracing reporter based

19   on combined transcription and alternative splicing regulatory elements to measure the frequency of

20   MET-like events during tumor growth and metastasis in the post-EMT Dunning rat AT3 model of

21   prostate cancer. The combinatorial use of both transcription and alternative splicing regulation provided

22   exquisite cell-type discrimination in the expression of enzymes, such as the Cre recombinase. Using the

23   lineage tracing reporter system, we were able to quantify, for the first time, overall frequencies of even

24   temporary MET during primary tumor and metastatic growth. Remarkably, we observed that the

1   frequency of MET within primary AT3 tumors and metastatic nodules was not significantly different,

2   with very low rates of MET taking place during the growth of tumors and metastases. In a parallel set of

3   experiments, we also used the combinatorial control strategy to drive a suicide reporter that kills cells

4   undergoing MET to test the hypothesis that MET is required for metastatic colonization in AT3s, CS-99

5   human uterine carcinosarcoma cells, and the mixed epithelial/mesenchymal human prostate

6   adenocarcinoma cell line DU145. Consistent with the MET frequency data, targeted killing of cells

7   undergoing MET did not reduce the number of macrometastatic colonies in the lungs using the AT3 or

8   DU145 model systems. Surprisingly, CS-99 cells harboring the suicide MET reporter led to dramatic

9   reductions in metastases compared to control CS-99 cells without the suicide reporter. Using gene

10   expression data from DU145 lung metastases that were serially passaged by intravenous (i.v.) injection,

11   we identified unique pathways linked to the MET-independent and MET-dependent metastatic

12   cascades. We also provide evidence for MET-independent metastasis using gene expression data from

13   metastatic bone biopsies in patients with metastatic, castration-resistant prostate cancer. This work

14   demonstrates the ability of tumors to metastasize efficiently via MET-dependent and MET-independent

15   routes.

16

17   **Results**

18   *Post-EMT AT3 cells undergo MET during tumor formation*

19   Previously, we developed a fluorescence-based reporter of MET, which was driven by epithelial-

20   specific skipping of exon IIIc from fibroblast growth factor receptor 2 (FGFR2) (Oltean et al., 2008; Oltean

21   et al., 2006; Somarelli et al., 2013; Wagner et al., 2004) (Figure 1A). The FGFR2 pre-mRNA undergoes

22   alternative splicing within its ligand binding domain to produce two mutually exclusive isoforms, IIIb and

23   IIIc. Epithelial cells include the IIIb exon while mesenchymal cells skip exon IIIb and include exon IIIc. The

24   RIIIcI[2] reporter contains the DsRed open reading frame (ORF) interrupted by the IIIc exon and flanking

1   introns (Wagner et al., 2004). Skipping of exon IIIc in epithelial Dunning rat prostate cancer DT cells leads

2   to an in-frame DsRed mRNA and expression of DsRed (Figure 1B; left column labeled 'DT+RIIIcI[2]'), while

3   inclusion of exon IIIc in mesenchymal AT3 cells interrupts the DsRed ORF, with little to no DsRed

4   expression (Figure 1B; second column). DsRed expression correlated with high E-cadherin staining and

5   low levels of vimentin in the epithelial DT cells while the mesenchymal AT3 cells expressed little to no

6   DsRed, low E-cadherin, and high levels of vimentin (Figure 1B).

7   Using this reporter, our group observed MET-like events within AT3 mesenchymal rat prostate

8   tumors and metastases (Oltean et al., 2006); however, these studies were limited by the lack of a tumor-

9   specific marker or label as a denominator to determine the amount of MET within the primary tumors.

10   To quantify the number of cells that undergo MET within AT3 primary tumors, we labeled AT3 cells with

11   RIIIcI[2] and Gint, the latter of which contains the EGFP ORF interrupted by a constitutively spliced intron.

12   The Gint reporter allows labeling of all tumor cells, while RIIIcI[2] reports on cellular phenotype (epithelial

13   or mesenchymal) at a specific moment in time. Subcutaneous injection of 5×10^5 AT3 cells harboring

14   both reporters led to growth of tumors within approximately 4-6 days. Tumors were harvested after

15   eight days, sectioned using a cryotome, and sections were analyzed by epifluorescence microscopy. As

16   we previously reported (Oltean et al., 2006), we observed rare foci of MET within AT3 primary tumors

17   (Figure 1C). Interestingly, many of the EGFP+ and DsRed+ AT3 cells resided near the edges of tumors

18   within EGFP- portions of the tumors, which we interpret to be fibroblasts and other cells from the host

19   that encapsulate the tumors (Figure 1C; bottom row; white arrows). Using flow cytometry on cell

20   suspensions from resected tumors (n = 6), we calculated the frequency of MET at a specific moment

21   during tumor growth by dividing the number of DsRed+ cells by the number of EGFP+ cells. We counted

22   an average of 91,489 EGFP+ events per tumor and estimated that an average of 262 of these EGFP+ cells

23   also express DsRed, indicating that MET is a rare event (0.28% +/- 0.04%) within AT3 primary tumors.

24

1   *Combinatorial control of reporters enables fine tuning of expression*

2   Although the RIIIcI[2] reporter provides unique information about the proportion of cells

3   undergoing MET at a specific moment in time, we postulated that transitions between epithelial and

4   mesenchymal states among highly aggressive cancer cells could be highly reversible, and we sought to

5   quantify the overall transition frequency during tumor progression. To accomplish this, we developed a

6   lineage tracing reporter based on Cre recombinase. Considering that extremely low levels of Cre

7   recombinase can trigger removal of genetic elements flanked by *LoxP* sites, we hypothesized that the

8   alternative splicing-based reporters may require additional control elements to enhance specificity of

9   expression. To test this hypothesis, four luciferase reporters were constructed. FFint, FFIIIcI[2], E-cadFFint,

10   and E-cadFFIIIcI[2] (Supplementary Figure 1A). As hoped, the combinatorial use of promoter and splicing

11   elements in E-cadFFIIIcI[2] had a multiplicative effect, providing over 50-fold higher expression of Firefly

12   luciferase in epithelial DT cells compared to mesenchymal AT3 cells (Supplementary Figure 1B). Perhaps

13   more importantly, the expression of Firefly luciferase in mesenchymal AT3 cells was very low, indeed

14   barely above background. These assays validated the combinatorial use of transcriptional and post-

15   transcriptional control elements to provide remarkable specificity among cell types and formed the basis

16   for the use of highly sensitive enzymatic reactions as reporters of cell fate and phenotype.

17

18   *Design and validation of lineage tracing reporters to track MET during tumor progression* in vivo

19   Validation of the combinatorial reporters indicated that multiple regulatory elements provided

20   enhanced specificity, which is critical for faithful reporter readout. As a result, the lineage tracing MET

21   reporters were generated based on the design of the E-cadFFIIIcI[2], in which the Firefly ORF was replaced

22   by the Cre recombinase ORF to give E-cadCreIIIcI[2]. Mesenchymal cells should not activate the E-cadherin

23   promoter and should include exon IIIc, leading to very low, if any, production of Cre (Figure 2A).

24   Conversely, epithelial cells express Cre via activation of the E-cadherin promoter and exon IIIc skipping

1   (Figure 2A). The E-cadCreIIIcI[2] reporter acts on a second plasmid, RG (De Gasperi et al., 2008), in which

2   the DsRed ORF containing a stop codon is flanked by *LoxP* sites followed by the EGFP ORF containing a

3   stop codon (Figure 2A). Expression of Cre during MET should lead to permanent removal of DsRed by

4   recombination at *LoxP* sites and consistent expression of EGFP.

5   To provide proof-of-concept that the lineage tracing reporters would accurately reflect cellular

6   phenotype, DT and AT3 cells harboring RG were transfected with an empty vector control, a plasmid

7   that constitutively expressed Cre recombinase, or E-cadCreIIIcI[2], and imaged by epifluorescence

8   microscopy. As expected, cells transfected with the pcDNA6 vector remained DsRed+, while both DT and

9   AT3 cells expressing the Cre ORF (Cre ORF) contained EGFP+ cells (Figure 2B). Persistence of DsRed+ cells

10   observed in all wells was due to incomplete transfection efficiency. Importantly, however, only the

11   epithelial DT cells expressed EGFP when transfected with E-cadCreIIIcI[2] whereas the mesenchymal AT3

12   cells transfected with E-cadCreIIIcI[2] were EGFP- (Figure 2B). Flow cytometric analysis of DTs and AT3

13   cells transfected with pcDNA6, Cre ORF, or E-cadCreIIIcI[2] confirmed the results observed by

14   epifluorescence microscopy, except that the more sensitive flow cytometry identified a small sub-

15   population (~8%) of EGFP+ AT3 cells (Figure 2C). Analysis of transcript abundance by qRT-PCR revealed

16   that DT cells containing E-cadCreIIIcI[2] expressed nearly 10-fold more Cre mRNA than AT3 cells with E-

17   cadCreIIIcI[2] (Figure 2D). Taken together, these results indicate that the lineage-tracing reporters

18   accurately reflected phenotypic status of epithelial and mesenchymal cells.

19

20   *Quantifying the frequency of MET during tumor growth and metastasis*

21   The data above suggested that the E-cadCreIIIcI[2] reporter could be used to determine the

22   frequency of MET events in tumors. AT3 cells stably transfected with RG and E-cadCreIIIcI[2] were injected

23   subcutaneously (s.c.) into the left flanks of male Copenhagen or nude rats, and primary tumors were

24   collected eight days later for analyses. For AT3 cells transfected with RG and pcDNA6 or E-cadCreIIIcI[2],

1   DsRed+ AT3 cells were sorted immediately prior to injections. For AT3 cells transfected with RG and the

2   constitutively active Cre ORF, EGFP+ cells were sorted immediately before injections. As observed with

3   AT3 cells in culture, tumors from cells transfected with pcDNA6 or Cre ORF reporters exclusively

4   expressed DsRed or EGFP, respectively (Figure 3A). Interestingly, tumors from cells harboring RG and E-

5   cadCreIIIcl[2] were predominantly DsRed+, but contained multiple foci of EGFP+ cells (Figure 3A; white

6   arrows).

7   Cells cultured from tumor explants maintained their fluorescence expression profiles for over 10

8   days of culture (Figure 3A; columns labeled "cultured cells"); cells from tumors containing pcDNA6 or

9   Cre were DsRed+ and EGFP+, respectively, while both DsRed+ and EGFP+ cells were observed from

10   tumors containing E-cadCreIIIcl[2] (Figure 3A; bottom right panels). The morphology of the EGFP+ cells

11   cultured from E-cadCreIIIcl[2] tumor explants appeared to be mesenchymal, which prompted us to

12   investigate whether these cells, which had undergone MET at some point during tumor growth, had

13   transitioned back to a mesenchymal phenotype. To do this, sections from E-cadCreIIIcl[2] tumors were

14   stained with the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and fibronectin.

15   EGFP+ cells exhibited a mesenchymal pattern of biomarker expression, with low E-cadherin and high

16   levels of N-cadherin and fibronectin (Figure 3B), suggesting that the MET-like event that led to activation

17   of Cre and a switch from DsRed to EGFP were transient and that these cells had either transitioned back

18   to a mesenchymal phenotype or had only activated an epithelial-like program long enough to express

19   Cre, but not alter cellular morphology and robustly express E-cadherin. We also hypothesized that

20   EGFP+ cells may be more stem-like, with a greater ability to toggle from one phenotypic state to

21   another; however, EGFP+ tumor cells did not express higher levels of the stem cell markers CD133 or

22   CD44. It should be noted that the entirety of AT3 tumor sections were strongly positive for these stem

23   cell markers, suggesting that the AT3 cells, as a population, are stem-like. The expression of stemness

1   markers in AT3 tumors is also consistent with the rapid development of AT3 anchorage-independent

2   spheroids in soft agar (Schaeffer et al., 2014).

3   Previous studies using the RIIIcl[2] reporter indicated that MET was observed more frequently in

4   lung micrometastases than lymph micrometastases, suggesting that some aspect of the lung

5   microenvironment or circulation in the bloodstream either mediated MET or required MET for seeding

6   of micrometastases. Therefore, we hypothesized that if MET was essential for metastatic colonization of

7   the lungs, then the number of cells that underwent an MET-like event would be significantly higher in

8   the lungs than in the primary tumors. It should be noted that AT3 tumors derived from s.c. injection

9   result in micro and macro metastases in the lungs (Oltean et al., 2006; 2008), but lung invasion can be

10 accelerated by injection directly into the circulation. AT3 cells with RG and E-cadCreIIIcl[2] were injected

11 via the tail vein (t.v.), and lungs were harvested after eight days. To our surprise, we observed EGFP+

12 AT3 cells only rarely within lung macrometastases (median = 1.25% EGFP+ cells; Figures 3C and 3D)

13 suggesting that the large majority of these metastases formed from cells that had never undergone

14 MET. AT3 cells cultured from lung explants were also predominantly DsRed+ (Figure 3C), with only one

15 EGFP+ colony growing out from the lung explants during the course of these experiments (data not

16 shown).

17   We next sought to quantify the frequencies of MET within cultured cells, primary tumors, and

18 metastases to determine whether the number of cells undergoing MET was higher in metastases than in

19 primary tumors. Cells from AT3 cultures (n = 3), primary tumors via s.c. injections (n = 14), and lung

20 metastases via t.v. injections (n = 11) were collected and analyzed by flow cytometry. There were

21 significantly more MET events in cultured cells than primary tumors (Figure 3D; p = 0.015), which implies

22 that there may be selective pressure for AT3 tumors to remain mesenchymal during *in vivo* tumor

23 growth. Perhaps more interestingly, the percentage of MET-like events in primary tumors and lung

24 macrometastases was similar (Figure 3D), with over 95% of cells having never undergone MET during

1   metastatic colonization in the lungs. We noted that the overall frequency of MET in lungs was more

2   variable than that observed in primary tumors, which could be due to more variability in

3   microenvironmental signals within the lungs or during circulatory transit as compared to primary

4   tumors. These data indicated that the overall frequency of MET in AT3 cells was not higher among those

5   that became resident in the lungs.

6   To verify that lung metastases were indeed mesenchymal, cryosections from AT3 lung

7   metastases labeled with Gint were stained with E-cadherin and vimentin. Metastases showed no E-

8   cadherin staining, despite strong E-cadherin staining in adjacent healthy lung tissue (Figure 3E). Lung

9   metastases were positive for vimentin, while the adjacent healthy lung tissue had very few vimentin-

10   positive regions (Figure 3E). Cultured cells from the lung metastases were also negative for E-cadherin

11   and positive for vimentin and fibronectin (Figure 3F). Cultured DT cells were used as a control for E-

12   cadherin staining, and cultured AT3 cells were used as a negative control for E-cadherin staining and a

13   positive control for vimentin and fibronectin (Figure 3F). The results from the MET reporters and

14   staining indicate that MET is a rare event during both AT3 tumorigenesis and formation of metastatic

15   colonies in the lungs.

16

17   *MET is not required for metastatic colonization of AT3 cells*

18   The MET reporters, along with staining of metastatic colonies, strongly suggested that MET was

19   not required for metastatic invasion of the lungs. Yet, based on the experiments above, we cannot rule

20   out that the rare MET events observed influenced the process and were required for colonization of

21   metastases. To test whether or not the formation of macrometastases requires MET, we designed a

22   "suicide reporter" in which MET triggers cell death via expression of an attenuated form of the A chain

23   of diphtheria toxin (DipA). The reporter, E-cadDipIIIcI[2], is the same as E-cadCreIIIcI[2], except that the Cre

1   ORF is replaced by a modified DipA ORF. Importantly, the A chain of diphtheria toxin is unable to cross

2   cell membranes (Breitman et al., 1990), which eliminates the potential for bystander effects.

3   To test the suicide reporters, DT and AT3 cells were transfected with either an empty vector

4   control (pcDNA6) containing a blasticidin resistance gene or E-cadDipIIIcI^2, which also contains a

5   blasticidin resistance gene. Two days after transfection, cells were treated with blasticidin, and

6   monitored for the outgrowth of colonies. After six days of blasticidin selection, all mock transfected DT

7   and AT3 cells were killed, while both cell types transfected with an empty vector that conferred

8   blasticidin resistance grew out efficiently (Figure 4A). There was, however, a remarkable difference

9   between DT and AT3 cells transfected with E-cadDipIIIcI^2. While there were very few, if any, DT colonies,

10   many AT3 colonies were detected (Figure 4A). Notably, AT3 cells containing the suicide reporter grew

11   out at a slower rate than AT3 cells harboring an empty vector, likely due to the toxicity of even minute

12   levels of DipA expression.

13   Selection for stable integrants was quantified using the WST-1 cell proliferation reagent and

14   normalized to the absorbance from mock-transfected cells. Both DTs and AT3 cells transfected with the

15   empty vector control grew out quickly during selection, albeit at different rates (Figure 4B). DT cells

16   transfected with the suicide reporter were negative when normalized to untransfected wells, which may

17   be due to the combined toxicity of blasticidin and DipA expression in these cells (Figure 4B). AT3 cells

18   transfected with the suicide reporter, on the other hand, produced colonies within four days of

19   blasticidin selection (Figure 4B).

20   AT3 cells containing Gint were stably transfected with either RIIIcI^2 or E-cadDipIIIcI^2, and 1 x 10^6

21   cells injected into nude rats via the tail vein. After 12 days, lungs were harvested and tested for the

22   presence of the E-cadDipIIIcI^2 reporter in the metastases using a PCR assay. We were able to confirm the

23   presence of the E-cadDipIIIcI^2 reporter within 3/4 of the AT3 lung metastases (Supplementary Figure

24   2A). Expression of DipA mRNA containing the IIIc exon in metastatic lung nodules was determined by RT-

1   PCR using a forward primer within the 5' DipA exon and a reverse primer within the IIIc exon. DipIIIc

2   mRNA was detected in 3/4 metastatic nodules, albeit at low levels when compared to AT3 E-cadDipIIIcI[2]

3   cells in culture (Supplementary Figure 2B). We attribute the lower levels of Dip transcripts in the

4   metastases compared to cells in culture to the much lower levels of RNA yield from the metastases.

5   Nonetheless, our results indicate that at least 75% of macrometastases harbored the DipA reporter at

6   the time of animal sacrifice and, perhaps more importantly, 75% of the metastases contained detectable

7   DipIIIc expression. These results indicate that the suicide reporter remained within the cells during

8   metastatic colonization.

9   To determine whether selective killing of cells undergoing MET reduced metastatic colonization

10   in the lungs, the numbers of macrometastases from cells with RIIIcI[2]/Gint or E-cadDipIIIcI[2]/Gint were

11   counted by epifluorescence microscopy at 40X total magnification. Figure 4C shows representative

12   images of metastases from RIIIcI[2]/Gint and E-cadDipIIIcI[2]/Gint cells. There was no qualitative difference

13   in the size or gross morphology of the metastases, with metastases from cells containing either the

14   control or suicide reporter forming round, multicellular, secondary lung tumors (Figure 4C). Importantly,

15   AT3 cells harboring RIIIcI[2]/Gint or E-cadDipIIIcI[2]/Gint formed equivalent numbers of metastases (Figure

16   4D). Overall, the results from lineage tracing reporters, suicide reporters, and biomarker staining clearly

17   indicate that MET was not required for metastatic colonization of the lungs in the mesenchymal AT3

18   prostate cancer model.

19

20   *Suicide reporters reveal distinct metastatic pathways*

21   We have noted a number of similarities between the AT3 tumors and prostate carcinosarcomas,

22   including their undifferentiated histology, mesenchymal-like gene expression profiles (Oltean et al.,

23   2008), aggressive nature, and preference for metastasizing to the lungs and lymph nodes (Oltean et al.,

24   2006). Indeed, AT3 cells resemble the more poorly differentiated component of human

1   carcinosarcomas in their histopathology, their likely epithelial origin and weak to negative co-expression

2   of epithelial biomarkers, such as cytokeratin, and expression of vimentin (Supplementary Figure 3). To

3   understand whether MET-independent metastasis is a property consistent across multiple

4   carcinosarcomas, we tested our suicide reporters in the CS-99 cell line, which was derived from a

5   human uterine carcinosarcoma and displays characteristics of the sarcomatous phenotype (Schulten et

6   al. 2008; 18334012). Morphologically, CS-99 cells exhibit a spindle-like morphology, but will form cell-

7   cell attachments at higher confluence (Figure 5A). CS-99s stably transfected with Rint, a plasmid

8   encoding DsRed interrupted by a constitutively spliced intron, express DsRed; however, CS-99s

9   transfected with RIIIcI^2 exhibit only low levels of DsRed expression (Figure 5A), which is consistent with

10   FGFR2 exon IIIc inclusion and a mesenchymal phenotype. We also characterized the expression of

11   endogenous FGFR2 isoforms in CS-99s by RT-PCR (Figure 5B). DT and AT3 cells served as controls for IIIb

12   and IIIc expression, respectively. Consistent with results from the RIIIcI^2 reporter, CS-99 cells exclusively

13   express FGFR2-IIIc mRNA (Figure 5B). CS-99s lack E-cadherin and express vimentin (Figure 5C). Epithelial

14   LNCaP and mesenchymal PC-3 cells were included for reference (Figure 5C). Together, these data verify

15   the mesenchymal-like phenotype and biomarker expression of the CS-99 cell line.

16   To ask whether CS-99 carcinosarcoma cells require MET during metastasis, we stably

17   transfected the E-cadDipIIIcI^2 suicide reporter followed by Gint, which marked these cells with EGFP.

18   Prior to performing *in vivo* experiments, we noted that approximately 50% of cells stably transfected

19   with E-cadDipIIIcI^2 lacked the E-cadDipIIIcI^2 reporter (data not shown). As a result, we sorted single cells

20   via FACS and selected by RT-PCR analysis three clones (clone 23, clone 27, and clone 33) that contained

21   E-cadDipIIIcI^2. Because the CS-99 cell line had never been used in a tail vein metastasis model and as

22   such, we had no indication of the metastatic penetrance *in vivo* we designed our tail vein injections to

23   include CS-99 cells that lacked the E-cadDipIIIcI^2 reporter. These control cells were distinguished from E-

24   cadDipIIIcI^2/Gint cells because they were stably transfected with Rint reporter and expressed DsRed, but

1   not EGFP. This strategy controls for any technical issues related to the tail vein injections. Using this

2   design, we were able to interrogate the importance of MET in metastasis, focusing on those animals for

3   which the tail vein injections worked properly. First, we stably transfected CS-99s with Rint and sorted

4   DsRed-expressing cells by FACS. Next, to control for the presence of Gint in E-cadDipIIIcI^2/Gint cells, we

5   also transfected Gint and sorted DsRed+/Gint- cells by FACS. A total of 5 x 10^5 of these DsRed+/Gint-

6   cells were co-injected with 5 x 10^5 of each E-cadDipIIIcI^2/Gint+ clone via the tail vein of Balb/c nu mice (n

7   =3 for clone 23, n = 3 for clone 27, and n = 4 for clone 33). After four weeks, 5/10 animals that were co-

8   injected with control cells and E-cadDipIIIcI^2/Gint+ cells had lung metastases. Strikingly, of the five

9   animals with visible Rint+ metastases, only one Gint+ metastasis was observed (Figure 5D). All other

10   animals completely lacked E-cadDipIIIcI^2/Gint+ cells, despite the presence of between one and 276

11   metastases from control Rint+/Gint- cells (Figure 5D).

12   We asked whether the difference in metastatic efficiency between control cells and those

13   harboring the E-cadDipIIIc^2 was due to a difference in growth rates. WST-1 cell proliferation assays

14   indicated that the E-cadDipIIIcI^2/Gint clones and Rint+/pGint- cells grow at equal rates (Figure 5E). We

15   also hypothesized that growth in an anchorage-independent setting may be more similar to the *in vivo*

16   metastatic niche than monolayer culture. Based on this notion, we asked whether anchorage-

17   independent growth conditions would induce MET. We performed anchorage-independent growth

18   assays with equal mixtures of Rint+/Gint- cells and E-cadDipIIIcI^2/Gint+ clones. Contrary to the *in vivo*

19   experiments in which E-cadDipIIIcI^2/Gint+ cells died, E-cadDipIIIcI^2/Gint+ cells did not undergo MET

20   under anchorage-independent conditions and survived. Both Rint+/Gint- and E-cadDipIIIcI^2/Gint+ cells

21   formed anchorage-independent colonies with equal efficiency (Figure 5F). Together, these data indicate

22   that, unlike AT3 cells, CS-99 carcinosarcoma cells utilize an MET-dependent pathway to metastasize.

23   Our suicide reporter studies suggest that different pathways to metastasis may exist, including

24   an MET-dependent and an MET-independent pathway. To better understand the role of MET in

1   metastasis, we also performed tail vein injections of DU145 human prostate adenocarcinoma cells

2   harboring E-cadDipIIIcI²/Gint. The DU145 line features a predominantly mesenchymal phenotype, but

3   has been shown to contain a small sub-population of epithelial-like cells (Putzke et al. 2011; 21703419).

4   In addition, others have shown that DU145 cells undergo MET when metastasizing to sentinel lymph

5   nodes of the prostate, but not when serially passaged through the prostate (Banyard et al.

6   2013; 24193225). To our surprise, unlike the CS-99s, but consistent with the AT3 model, DU145 E-

7   cadDipIIIcI² cells did not undergo MET when metastasizing to the lungs (Figure 6A). DU145 cells

8   harboring control reporters developed a median of 99 metastases while DU145 EcadDipIIIcI²/Gint cells

9   developed a median of 403 metastases. In keeping with the results from the suicide reporter, DU145 E-

10 cadDipIIIcI² lung metastases lacked E-cadherin and expressed vimentin (Figure 6B).

11   To further verify that DU145 cells do not undergo MET during metastatic progression, we

12 queried publicly available gene expression data from parental DU145s and i.v. injected lung metastases

13 (Banyard et al. 2013; GSE51755). Consistent with our suicide reporter results and IF staining, there was

14 no difference in expression of epithelial or mesenchymal biomarkers in DU145 metastases compared to

15 parental DU145s (Supplementary Figure 4A; white bars). Similarly, whole genome expression analysis

16 from n = 22 primary tumors and n = 33 bone marrow metastases from patients with prostate cancer

17 (Stanbrough et al. 2006; 16510604; GSE32269) showed significant downregulation of epithelial

18 biomarkers E-cadherin, keratin 8, and keratin 18 and significant upregulation of mesenchymal markers

19 SNAI2 (Slug), ZEB1, ZEB2, vimentin, MMP2, MMP9, and N-cadherin in metastatic samples compared to

20 primary tumors (Supplementary Figure 4B). Together, these analyses support the idea that metastasis

21 can occur via an MET-independent pathway.

22   Interestingly, we noted that i.v. passages three and four of DU145s through the lungs led to

23 significant upregulation of epithelial markers E-cadherin, TJP1 (ZO-1), and keratin 8 and significant

24 downregulation of the mesenchymal marker TGFB2 (Supplementary Figure 4A; gray bars). Using i.v.

1   serial passages one and two as a model for an MET-independent metastatic event and i.v. serial

2   passages three and four as an MET-dependent route to metastasis, we asked which pathways differed

3   between these two types of metastatic cascade. GO terms significantly over-represented and unique to

4   the MET-independent cascade included "cell cycle", "cell cycle process", "nucleus", and "cellular

5   macromolecular metabolic process". Similarly, GO terms significantly over-represented in the bone

6   metastases also included "cell cycle", "cell cycle process", and "nucleus" along with multiple additional

7   GO terms related to the G1/S and G2/M transitions. GO terms significantly over-represented and unique

8   to the MET-dependent cascade included "mitochondrial membrane part", "intracellular organelle part",

9   "cytoplasmic part", and "organelle part". Moreover, the GO terms found in the MET-dependent DU145

10 metastases were absent from the list of GO terms in the MET-independent bone metastases. These

11 observations suggest that the MET-independent metastases upregulate cell cycle genes while MET-

12 dependent metastases may express genes related to cellular metabolism. Together, our data reveal

13 distinct MET-dependent and MET-independent pathways that culminate in metastatic disease.

14

15

16 **Discussion**

17   During carcinoma progression, EMT is generally accepted as a means by which cancer cells gain

18 invasive capabilities and break free from the confines of the primary tumor (Hanahan and Weinberg,

19 2000; Thiery, 2002). Several investigations have also suggested that MET may be important for

20 metastatic colonization subsequent to dissemination from the primary tumor. For example, it is thought

21 that re-expression of E-cadherin by way of MET confers upon the disseminated cancer cell several

22 properties that may facilitate its survival within a new tissue, including activation of the AKT and

23 ERK/MAPK pathways and/or attachment to heterologous cells within the healthy tissue (reviewed in

24 (Wells et al., 2008)). Along these lines, Tsai et al. 2012 suggested that Twist1-induced EMT in a

1   chemically-induced model of squamous cell carcinomas lowered the proliferative potential of

2   metastases within the lungs (Tsai et al. 2012). In addition, Stankic et al. (2013; 24332369) showed that

3   ID1, a dominant negative regulator of TWIST1, facilitates breast cancer metastasis by inhibiting TWIST1

4   and inducing MET. Like these previous studies, the present work also suggests that CS-99 human

5   carcinosarcoma cells require MET to efficiently metastasize in the lungs. Yet, our data also indicate that

6   other, post-EMT cancers can metastasize through a mechanism that appears to be completely

7   independent of MET.

8   The finding that MET appears to be critically important for metastasis of some cancers, while

9   others have no apparent need for this transition is reminiscent of predictions by Brabletz (2012) that

10   two "routes to metastasis" exist. Brabletz postulates that some cancers will be plasticity-dependent

11   while other cancers will metastasize by way of one or more plasticity-independent pathways. Our data

12   provide evidence for both the MET-dependent and MET-independent routes to metastasis. Yet, it

13   remains unclear which genetic and/or environmental factors drive a given cancer type down either of

14   these pathways. AT3s and CS-99s, for example, both represent sarcomatoid-like models of

15   carcinosarcoma. Despite their similarities, AT3s metastasize efficiently via an MET-independent program

16   while CS-99s seem to use an MET-dependent metastatic cascade.

17   Unlike the carcinosarcoma models, DU145s are a classical model of aggressive prostate

18   adenocarcinoma. Perhaps unexpectedly, DU145s, like AT3 cells, also metastasize independently of MET.

19   Yet, DU145 cells are capable of transitioning to a more epithelial phenotype over successive rounds of

20   i.v. injection and metastasis to the lungs (Banyard et al. 2014; 24193225 and data herein). Analysis of gene

21   expression data from the mesenchymal-like and the epithelial-like DU145 metastases pinpointed

22   different pathways that may be responsible for driving each phenotype: upregulation of cell cycle genes

23   in the MET-independent metastases and upregulation of genes related to mitochondria and the electron

24   transport chain in the MET-dependent metastases. Interestingly, dysregulation of both the cell cycle and

1   mitochondrial function have been linked to metastatic progression. For example, Basak et al. (2008;

2   18471976) identified phosphatase of regenerating liver-3 (Prl-3) as a p53-dependent cell cycle regulator

3   that is involved in metastatic progression of colorectal cancer (11598267; 12782572).   In addition,

4   microarray analyses from metastatic melanoma indicated that progression to metastatic disease was

5   correlated with upregulation of both EMT and cell cycle programs (reviewed by Danilov et al., 2008;

6   18496651). It is noteworthy that metastatic melanomas mirror our observations in metastatic DU145s

7   that a link exists between metastatic propensity and pathways related to both EMT and the cell cycle.

8   Unlike MET-independent DU145 cells, MET-dependent DU145 cells increased expression of

9   genes related to mitochondrial function. Interestingly, Porporato et al. (2014; 25066121) reported that

10   migration, invasion, and metastasis were enhanced by mitochondrial dysfunction related to superoxide

11   production. In addition, knockdown of Complex I members GRIM-1 or NDUFS3 led to production of

12   reactive oxygen species and increased migration and invasion (He et al. 2013; 23630608). These changes

13   were also accompanied by an EMT-like phenotypic transition, which is contrary to the gene expression

14   changes observed in MET-like DU145s. Yet, despite this difference, it is interesting to point out that

15   DU145 MET-dependent metastases increased expression of multiple members of the NADH

16   dehydrogenase (ubiquinone) complex, or Complex I, which is responsible for catalyzing the transfer of

17   electrons from NADH to Coenzyme Q10. In the process, Complex I produces high quantities of

18   superoxide (Murphy, 2009; 19061483), and it is attractive to postulate that MET-dependent DU145s may

19   metastasize via elevated production of superoxide.

20   Collectively, our data provide experimental validation of initial predictions that cancers undergo

21   metastatic progression via plasticity-dependent and -independent mechanisms (Brabletz, 2012). We

22   demonstrated the presence of MET-dependent and MET-independent pathways using *in vivo* models of

23   carcinosarcoma and adenocarcinoma and gene expression data from bone metastases in prostate

24   cancer patients. Together, our data suggest that the same cancer type, and maybe even cells within a

1 single human tumor, may be capable of metastasizing via different routes. Perhaps most importantly,

2 these findings argue that effective treatment of metastatic disease may require treatments that target

3 different metastatic pathways.

4

5 **Experimental Procedures**

6 A detailed explanation of the experimental procedures is included as a supplementary file.

7

8 **Acknowledgements**

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1   **Figure Legends**

2

3   **Figure 1. Fluorescence-based MET reporters reveal epithelial plasticity in mesenchymal rat prostate**

4   **tumors. A.** To monitor epithelial plasticity during tumor growth, Dunning rat AT3 mesenchymal prostate

5   cells were stably transfected with two reporters, Gint and RIIIcI[2]. The Gint plasmid encodes the EGFP

6   ORF interrupted by a constitutively spliced adenoviral intron (dashed lines). The RIIIcI[2] reporter contains

7   the DsRed ORF interrupted by fibroblast growth factor receptor 2 IIIc exon and flanking introns. **B.**

8   Skipping of exon IIIc and flanking introns leads to fusion of the DsRed ORF and production of DsRed in

9   epithelial DT cells while inclusion of exon IIIc in mesenchymal AT3 cells prevents expression of DsRed.

10   Immunofluorescence staining shows that epithelial DT cells express high levels of the epithelial

11   biomarker E-cadherin with low vimentin, a mesenchymal marker. Mesenchymal AT3 cells, on the other

12   hand, have almost no E-cadherin, but robustly express vimentin. Hoechst stained cells to the left of each

13   panel. **C.** Frozen sections from AT3+Gint+RIIIcI[2] tumors reveal foci of DsRed expressing cells near the

14   tumor edge. Two representative sets of images are shown. The white box in the second row of images

15   indicates the region that is zoomed below each image. A dotted line indicates the border between the

16   inner tumor mass and the unlabeled cells of the tumor capsule. Arrows highlight tumor cells that reside

17   within the tumor capsule that have undergone MET. Scale bars = 50 µm unless otherwise specified.

18

19

20   **Figure 2. Design and validation of lineage tracing reporters of MET. A.** (Top) E-cadCreIIIcI[2] is identical to

21   other reporters described in Figures 1 and 2, except that the Cre recombinase ORF replaces a

22   fluorescent protein or luciferase ORF. In mesenchymal cells, the E-cadherin promoter is inactive and the

23   IIIc exon is included; no Cre is produced. In epithelial cells, Cre is actively transcribed from the E-

24   cadherin promoter and exon IIIc is efficiently skipped, leading to expression of Cre recombinase. The E-

1 cadCreIIIcI^2 reporter acts on RG, which contains a DsRed ORF and stop codon (red octagons) flanked by

2 *Loxp* sites (triangles) followed by the EGFP ORF and a stop codon. MET leads to activation of Cre

3 recombinase and a switch from DsRed to EGFP expression. **B.** Epithelial DT and mesenchymal AT3 cells

4 were stably transfected with RG followed by transfection with pcDNA6, a constitutively active Cre ORF,

5 or the E-cadCreIIIcI^2 MET reporter. Cells transfected with pcDNA6 exclusively expressed DsRed, and cells

6 harboring the Cre ORF activated EGFP expression. When transfected with the E-cadCreIIIcI^2 reporter,

7 only epithelial DT cells switched from DsRed to EGFP expression while AT3 cells maintained DsRed

8 expression. Scale bar = 50 μm. **C.** Flow cytometry analysis shows that a small sub-population of AT3 cells

9 undergo MET. **D.** RT-qPCR of DT and AT3 cells transfected with RG and E-cadCreIIIcI^2 reveals

10 approximately 10-fold higher expression of Cre in epithelial DT cells than mesenchymal AT3 cells.

11

12 **Figure 3. Lineage tracing suggests that MET is rare during tumorigenesis and metastasis. A.** Tumors

13 from AT3 cells harboring RG and either pcDNA6, constitutively active Cre, or E-cadCreIIIcI^2 express

14 DsRed, EGFP, and a mixture of both fluorescence proteins, respectively. Tumors with the MET reporter

15 contain small foci of EGFP-expressing cells (white arrows). We observed overall higher levels of DsRed

16 expression in tumors containing E-cadCreIIIcI^2 than those harboring pcDNA6 (note the difference in

17 intensity in the DsRed channel). Cultured cells from each tumor type are indicated in the right set of

18 panels. A mixture of DsRed-positive and EGFP-positive cells were cultured from tumors harboring the

19 MET reporter, E-cadCreIIIcI^2. B. Immunohistochemical staining of AT3+RG+E-cadCreIIIcI^2 tumor sections

20 suggests that MET is transient, with EGFP expressing cells returning to a mesenchymal phenotype.

21 Sections were stained with the epithelial marker E-cadherin, the mesenchymal markers fibronectin and

22 N-cadherin, and the stem cell markers CD133 and CD44. There was no relationship between EGFP

23 expression (MET) and the expression levels of stem markers CD133 or CD44. **D.** The percent MET in

24 cultured cells, primary tumors, and lung metastases was quantified by flow cytometry. There was a

1 significant difference in the %MET between cultured cells (+ symbol) and primary tumors (○ symbol);

2 however, there was no significant difference between the %MET in primary tumors and lung metastases

3 (□ symbols), with an average of >98% of cells never undergoing MET. **E.** Lung metastases (green) do not

4 express the epithelial marker E-cadherin despite robust expression of E-cadherin in adjacent healthy

5 lung (red). Similarly, AT3 lung metastases express high levels of the mesenchymal marker vimentin (red),

6 with almost no vimentin in adjacent healthy lung tissue. **F.** Cultured AT3 cells from lung explants have

7 low E-cadherin expression and high vimentin and fibronectin expression. Cultured epithelial DTs and AT3

8 cells were used as controls. Scale bars = 50 μm unless otherwise indicated.

9

10 **Figure 4. Suicide reporters selectively kill cells undergoing MET. A.** DTs and AT3 cells were left

11 untransfected or transfected with an empty vector (pcDNA6) or E-cadDipIIIcI^2 and subsequently selected

12 with blasticidin. All untransfected epithelial DT and mesenchymal AT3 cells are killed by blasticidin, while

13 DTs and AT3 cells transfected with an empty vector containing a blasticidin resistance marker survive

14 blasticidin selection. Importantly, only mesenchymal AT3 cells transfected with the E-cadDipIIIcI^2 suicide

15 reporter grow out during blasticidin selection while all epithelial DT cells with the reporter die. **B.**

16 Quantification of DT and AT3 growth during blasticidin selection by WST-1 cell growth assay. **C.**

17 Representative lung metastases from AT3 cells harboring RIIIcI^2+Gint or E-cadDipIIIcI^2+Gint. **D.** No

18 difference was observed in the number of macrometastases in each group (RIIIcI^2 vs. suicide reporter).

19

20 **Figure 5. CS-99 human carcinosarcoma cells require MET during metastatic colonization. A.** CS-99 cells

21 in monolayer cultures possess cell-cell contacts, with some spindle-shaped cells interspersed throughout

22 the culture. CS-99 cells transfected with a control reporter contains a constitutively spliced intron (Rint)

23 express DsRed while cells transfected with RIIIcI^2 express only background levels of DsRed. **B.** RT-PCR

24 indicates that CS-99 cells express FGFR2-IIIc. Epithelial DT and mesenchymal AT3 cells are included as

1   controls for the IIIb and IIIc isoforms, respectively. For each cell type, RT-PCR products are mock

2   digested (M), digested with the FGFR2 IIIb-specific enzyme, AvaI (A), or digested with the FGFR2 IIIc-

3   specific enzyme, EcoRV (E). Reactions with no reverse transcriptase are indicated by (-) for each cell

4   type. **C.** CS-99 cells lack E-cadherin and express vimentin. Epithelial LNCaP and mesenchymal PC3 cells

5   are included as controls for E-cadherin and vimentin, respectively. **D.** Rint+/Gint- CS-99 cells were mixed

6   with CS-99 clones harboring E-cadDipIIIcI[2] and Gint and injected into nude mice via the tail vein. The

7   number of DsRed+ and EGFP+ metastases in each animal was quantified by epifluorescence microscopy.

8   Each point represents the number of metastases from one animal. **E.** CS-99 Rint+/Gint- cells and E-

9   cadDipIIIcI[2]/Gint clones grow at equal rates in WST-1 cell proliferation assays. Statistically significant

10   differences between Rint+/Gint- and any of the three clones are indicated by asterisks. **F.** Soft agar

11   assays with CS-99 Rint+/Gint- cells and E-cadDipIIIcI[2]/Gint clones. Rint+/Gint- cells were mixed 1:1 with

12   E-cadDipIIIcI[2]/Gint clones and allowed to form colonies for two weeks. The number of colonies formed

13   by the control cells (Rint+/Gint-) or each E-cadDipIIIcI[2] clone is quantified in the bar graph. P-values are

14   indicated above the brackets. **G.** CS-99 metastases (DsRed+) lack E-cadherin (green).

15

16   **Figure 6. DU145 human prostate adenocarcinoma cells metastasize via an MET-independent pathway.**

17   **A.** Injection of DU145 E-cadCreIIIcI[2] (as a control) or E-cadDipIIIcI[2] cells led to formation of lung

18   metastases with 100% penetrance. There was no difference in the number of metastases between

19   control cells or cells with the suicide reporter. **B.** DU145 E-cadDipIIIcI[2] metastases lack E-cadherin

20   expression and stain positive for vimentin.

21

22   **Supplementary Figure 1. Combinatorial reporter control enhances discrimination between cell types.**

23   **A.** To test whether combinatorial control of reporters has an additive effect on segregating different cell

24   types, four constructs were created. The FFint and FFIIIcI[2] reporters are identical to Gint and RIIIcI[2],

1 respectively, except that fluorescent proteins are replaced by the firefly luciferase ORF. The E-cadFFint

2 and E-cadFFIIIcI^2 reporters are identical to FFint and FFIIIcI^2, respectively, except that the CMV promoter

3 is substituted for the E-cadherin promoter. **B.** All four reporters were transfected into epithelial DT and

4 mesenchymal AT3 cells along with a constitutively active *Renilla* luciferase plasmid. Splicing alone and

5 promoter control alone provide seven-fold and four-fold discrimination, respectively, between cell

6 types; however, the combined use of splicing and promoter control increases the difference in luciferase

7 expression to 53-fold. Bars not connected by the same number are significantly different.

8

9 **Supplementary Figure 2. Detection of the Diphtheria toxin gene and transcript in AT3 lung metastases.**

10 **A.** A total of 75% (3/4) AT3 metastatic lung nodules were positive for the E-cadDipIIIcI^2 reporter (black

11 arrow). M: 1Kb+ molecular weight marker (Invitrogen); -C: no template control; +C positive control (E-

12 cadDipIIIcI^2 plasmid); lanes 4-7: DNA from E-cadDipIIIcI^2 lung metastases. **B.** RT-PCR of exon IIIc-

13 containing DipA transcripts in AT3 metastatic lung nodules. A total of 75% (3/4) of lung

14 macrometastases expressed DipIIIc transcripts (black arrows). M: molecular weight marker (same as E

15 above); -C: negative control (AT3 cultured cells containing RIIIcI^2); +C: positive control (AT3 cultured cells

16 containing E-cadDipIIIcI^2); lanes 6-15: AT3 metastatic lung nodules.

17

18 **Supplementary Figure 3. Histopathologic comparison of AT3 primary tumors and human**

19 **carcinosarcomas. A.** Hematoxylin and eosin staining of human carcinosarcomas and AT3 primary tumors

20 revealed that both tumors are poorly-differentiated while many areas show lack of epithelial

21 differentiation on H&E stains. Images from each of three human carcinosarcoma samples are shown.

22 Like the AT3 tumors, the human carcinosarcoma specimens are poorly-differentiated, with frequent foci

23 of necrosis, high mitotic and apoptotic rates, and extensive invasion of adjacent structures. Yet, unlike

24 the AT3 tumors and metastases, all three human tumors appeared to be biphasic, with part of the

1   human tumors having a more spindled cytology and a fasciculated architecture suggestive of

2   mesenchymal differentiation; other areas of these neoplasms were more poorly-differentiated, with

3   features more like those of the AT3 cell line-derived tumors (vesiculated nuclei with nucleoli, and

4   abundant cytoplasm). **B.** AT3 tumors were negative for pan-cytokeratin staining; two of the primary

5   human tumors showed weak, focal cytokeratin staining in the more poorly-differentiated areas and the

6   third was negative for cytokeratin. The human primary carcinosarcomas also displayed patchy vimentin

7   staining throughout, as did the AT3 primary tumors. These data suggest that the AT3 tumors most

8   closely resemble the poorly-differentiated component of carcinosarcomas of the prostate as opposed to

9   adenocarcinoma of the prostate, in which epithelial histology is predominant.

10

11   **Supplementary Figure 4. Validation of MET-independent metastases in gene expression datasets. A.**

12   DU145 human prostate cancer cells serially passed *in vivo* through the lungs by intravenous injection

13   show no significant change in EMT/MET biomarkers (white bars). By serially passages three and four,

14   however, DU145 metastases express higher levels of epithelial biomarkers E-cadherin (CDH1), keratin 8

15   (KRT8), and keratin 18 (KRT18) and lower levels of the EMT inducer transforming growth factor beta

16   (TGFB2). **B.** Bone metastases from patients with prostate cancer have significantly lower levels of CDH1,

17   KRT8, and KRT18, and significantly higher expression of numerous mesenchymal biomarkers, including

18   Slug (SNAI2), Zeb1, Zeb2, vimentin (VIM), MMP2, MMP9, and CDH2. Genes that were unavailable in the

19   data set are indicated by N/A. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

A

Gint

CMV — E — GFP

RIIIcl² — CMV — Ds — IIIc — Red

B

	Phase	DsRed Channel	E-cadherin	Vimentin
DT				
AT3				

C

Hoechst | EGFP Channel | DsRed Channel

Tumor

Tumor capsule

20 μm

A

B

	DT		AT3	
pcDNA6				
Cre ORF				
E-cadCreIIIcI[2]				

C

DsRed vs EGFP

	pcDNA6	Cre ORF	E-cadCreIIIcI[2]
DT	100% / 0%	78% / 22%	66% / 34%
AT3	100% / 0%	7% / 93%	92% / 8%

D

Cre Expression Fold Change (normalized to U1 snRNA)

p = 0.034

DT ≈ 1.0, AT3 ≈ 0.09

A

Tumors | Cultured cells

pcDNA6 — DsRed Channel / EGFP Channel / DsRed Channel / EGFP Channel

Cre

E-cadCreIIIcl²

B

Hoechst | DsRed Channel | EGFP Channel | E-cadherin

Fibronectin

N-cadherin

CD133

CD44

C

Lung metastasis

Lung metastasis

Cultured from lung

D

% Mesenchymal-Epithelial Transitions

+ Cultured cells
○ Primaries
▪ Metastases

n.s.

p = 0.015

E

	Hoechst	Metastases-EGFP	MET marker	Merge
			E-cadherin	
			Vimentin	

F

	E-cadherin	Vimentin	Fibronectin
DT			
AT3			
Tumor			
Lung			

A

Vis | Rint | RIIIcl²

B

DT				AT3				CS-99			
M	A	E	-	M	A	E	-	M	A	E	-

C

	LNCaP	PC3	CS-99
E-cadherin			
β-actin			
Vimentin			
PTBP1			

D

p<0.001

276 →

Number of metastases

Rint+/Gint−   E-cadDipIIIcl²

E

- Rint
- Clone 23
- Clone 27
- Clone 33

Absorbance (450 nm)

Time (hrs)

F

Rint | Rint | Rint | Rint
Rint | Clone 23 | Clone 27 | Clone 33

■ EGFP colonies
□ DsRed colonies

p<0.001

Number of colonies

Rint   Clone 23   Clone 27   Clone 33

A

B

Hoechst | Metastasis-EGFP | E-cadherin | Vimentin

Supplemental Experimental Procedures

Cloning and expression of reporters

Both the RIIIcI^2 (Wagner et al., 2004) and Gint (Bonano et al., 2007) reporters have been described previously. To construct the lineage tracing reporters, the DsRed ORF from RIIIcI^2 was replaced with the Cre ORF. To do this, the Cre ORF was subcloned from pBS185 (Addgene plasmid 11916; (Sauer and Henderson, 1990)) separated into two pseudo-exons, with the first Cre pseudo-exon containing amino acids 1 to 161 (bp 1 to 483) of the ORF and the second Cre pseudo-exon containing amino acids 162 to 343 (bp 484 to 1032) of the ORF. Cre pseudo-exon one was inserted via HindIII and XbaI, and pseudo-exon two was inserted using ApaI and AgeI. The CMV promoter of RIIIcI^2 was then replaced with bp 638 to 1028 of the human E-cadherin promoter to create E-cadCreIIIcI^2. The Cre ORF was subcloned from pBS185 into pcDNA6 V5/His A via NheI and XhoI sites. The RG plasmid (a gift of Dr. Rita DeGasperi) was subcloned into pcDNA3.1+ (G418) downstream of the CMV promoter via 5' NheI and 3' ApaI sites. The E-cadDipIIIcI^2 reporter was created in a similar manner to that of E-cadCreIIIcI^2. The Diphtheria toxin A (DipA) pseudo-exon one consists of amino acids 1-24 (bp 1-72), and pseudo-exon two is comprised of amino acids 25-193 (bp 73-588). DipA pseudo-exons one and two were subcloned into pcDNA6 V5/His A via HindIII and XbaI and ApaI and NotI, respectively. The FFint and FFIIIcI^2 reporters have been described previously (Oltean et al., 2006). E-cadFFint and E-cadFFIIIcI^2 were constructed by replacing the CMV promoter from FFint and FFIIIcI^2 with the E-cadherin promoter described above (a kind gift of Drs. Jennifer Yori and Ruth Keri; (Yori et al., 2010)) using KpnI and SacI sites. RIIIcI^2, Gint, pRG, E-cadCreIIIcI^2, and E-cadDipIIIcI^2 were transfected into cells using Lipofectamine 2000 following standard protocols and stable integrants were selected with 500 µg/ml blasticidin (RIIIcI^2, E-cadCreIIIcI^2, and E-cadDipIIIcI^2) or 500 µg/ml G418 (Gint and RG). FFint, FFIIIcI^2, E-cadFFint, and E-cadFFIIIcI^2 were transiently transfected along with a control *Renilla* luciferase plasmid, pRL-TK, using Lipofectamine 2000. After 48 hours, cells were lysed in 1X passive lysis buffer (Promega) and analyzed

for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). Samples were read in a Lumat LB 9507 luminometer (Berthold).

Tumor cell injections and processing of primary and metastatic tumors

On the day of injections, cells were sorted by flow cytometry to gate away any negative cells. Subsequent to sorting, cells were centrifuged at 250 x g for five minutes and resuspended in PBS at a concentration of 5×10^5 cells/200 µl. A total of 5×10^5 AT3 cells were injected subcutaneously into the left flanks of Copenhagen or nude rats (Charles River). Nude rats were used only when Copenhagen rats were unavailable. For tail vein injections, cells were processed in the same way, except that 1×10^6 AT3 cells/200 µl were injected. After eight days, primary tumors or lungs were harvested, and tissues were processed for further analyses. DU145 tail vein injections were performed as described above with 5×10^6 cells/200 µl injected into Balb/c nu mice. CS-99 tail vein injections included 5×10^5 Rint+/Gint- cells and 5×10^5 E-cadDipIIIcI2/Gint+ cells in 200 µl injected into Balb/c nu mice. For cryosectioning and staining, tissues were placed into cryomolds filled with O.C.T. compound (VWR), flash frozen in liquid nitrogen, and stored at -80°C until use. To derive AT3 cells from primary tumors and metastatic nodules, ~4 mm^3 pieces of primary tumor or lung were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Media was replaced each day for five days, after which the tissues were removed, and cultures were maintained for an additional two to five days prior to fixation and imaging. For RNA and DNA extraction, lung metastases were stored in RNA Later at -80°C or frozen at -20°C, respectively until use.

Immunofluorescence staining, flow cytometry, and imaging

Tumor and lung cryosections (18 µM) were prepared using a Microm HM 550 cryotome and affixed to glass slides. Sections were marked with a hyodrophobic pen, fixed in 4% paraformaldehyde for

15 minutes, washed in phosphate buffered saline (PBS), blocked one hour in 5% bovine serum albumin (BSA) in PBS at room temperature, and incubated in the presence of primary antibody diluted in 5% BSA in PBS overnight at 4°C in sealed containers with moist paper towels in the corner of each dish. The next day, slides were washed with PBS, incubated in the presence of a 1:2,000 dilution of appropriate AlexaFluor 674 secondary antibody for one hour at room temperature in the dark, washed once more in PBS, and incubated for five minutes at room temperature in Hoechst stain diluted 1:2,000 in PBS. Slides were washed three times in PBS, and fluorescence images were captured using an Olympus IX 71 epifluorescence microscope with a DP70 digital camera and processed with CellSens software (Olympus). Cell staining followed the same procedure, except that all steps were performed in tissue culture plates. The following antibodies and dilutions were used: mouse anti-E-cadherin (BD Biosciences; cat. #610181; 1:1,000), rabbit anti-E-cadherin (Cell Signaling; cat. #3195S; 1:250), mouse anti-vimentin (ABD Serotec; cat. #MCA862; 1:500), mouse anti-cellular fibronectin (Sigma; cat. #F6140; 1:500), mouse anti-N-cadherin (BD Biosciences; cat. #610921; 1:1,000), rabbit anti-CD133 (Santa Cruz; cat. #sc-30219; 1:1,000), rabbit anti-CD44 (Santa Cruz; cat. #sc-7946; 1:1,000), mouse anti-β-actin (Santa Cruz; cat. #sc-47778; 1:4000), and rabbit anti-PTBP1 (Wagner et al. 1999; 1:4000). Formalin fixed and paraffin embedded (FFPE) blocks from AT3 primary tumors and lung metastases were generated by the Duke University Research Histology Laboratory. Human carcinosarcoma blocks were provided by Jonathan I. Epstein, Johns Hopkins Hospital. All FFPE blocks were sectioned and stained with Hematoxylin and eosin or immunostained by the Duke University Research Histology Laboratory. Histological characterization of AT3 tumors and lung metastases and human carcinosarcomas was performed by a pathologist (AFB) blinded to the identity and location of the malignancies . Flow cytometric analysis was performed by the Duke University Flow Cytometry Core Facility, and samples were analyzed using the WinMDI software (version 2.9).

Nucleic acid extraction and PCR

DNA was extracted from frozen lung metastases using the DNeasy Blood and Tissue kit (Qiagen) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). A total of 200 ng of genomic DNA from each lung metastasis was used in PCR containing 1X PFU Ultra AD buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 1.25 U of PFU Ultra AD (Agilent), and nuclease-free water in a 50 µl reaction volume. Thermocycling conditions were as follows: 95°C for two minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 68°C for two minutes, with a final extension of 68°C for 10 minutes. Samples were electrophoretically separated in 1% w/v agarose-Tris/acetate/EDTA gels and imaged using a ultraviolet light box affixed with a Canon Rebel XS camera. Primers to detect DipA within genomic DNA of lung metastases were: 5'- CCT CAG ACT AAA CCT GGT TAT GTA GAT TCC ATT C -3' and 5'- GAT TTC CTG CAC AGG CTT GAG CCA TAT AC-3'. To extract RNA from lung metastases, tissues were flash frozen with liquid nitrogen and ground to a powder with a mortar and pestle. To isolate RNA from cultured cells, cells were trypsinized in the presence of 0.05% trypsin and centrifuged at 250 x g for five minutes. RNA was extracted from the metastatic tissue and cell pellets using Qiashredder homogenizers and the RNeasy Mini Kit (Qiagen). Reverse transcription reactions were comprised of 1 µg of RNA 200 ng of random hexamer primers, 1X IMPROMII reverse transcriptase buffer, 10 µM dNTPs, 3.75 mM $MgCl_2$, 0.1 µl RNasin, and 1 µl of IMPROMII reverse transcriptase in a total volume of 20 µl. PCR conditions were the same as those described above. Primers to detect Cre were: 5'-GAA CCT GAT GGA CAT GTT CAG G-3' and 5'-AGT GCG TTC GAA CGC TAG AGC CTG T-3'. Primers to detect exon IIIc-containing DipA transcripts were: 5'- GGG CGC TGA TGA TGT TGT TGA TTC-3' and 5'-TAC GCT TAA CGC TTT CGC CTG TTC-3'. FGFR2 isoforms were detected in DT and AT3 cells by RT-PCR as previously described (Somarelli et al., 2013). The same protocol was used to monitor expression of FGFR2 isoforms in CS-99s, except the following

human primers to detect FGFR2 were used: 5'-GCA AGG TTT ACA GTG ATG CCC AGC C-3' and 5'-GGA TGA CTG TTA CCA CCA TAC AGG CGA T-3'.

Validation of E-cadDipIIIcI[2] reporter by WST-1 assays

A total of 3×10^5 DT or AT3 cells were transfected with pcDNA6/V5/HisA or the E-cadDipIIIcI[2] reporter using the method described above. The next day, 1×10^3 cells were seeded into individual wells of 96-well plates and incubated in the presence of blasticidin after an additional 24 hours of growth. Cell growth was measured each day by adding 10 µl of WST-1 (Roche) to each well and incubating for 30 minutes at 37°C. Plates were read at 450 nm on a Biotek plate reader. Cell growth was normalized to untransfected cells treated with blasticidin. Growth curves for CS-99 E-cadDipIIIcI[2] clones were obtained by seeding 1×10^4 cells into individual wells of 96-well plates with six replicates per clone per time point. Plates were read as described above.

Anchorage-independent growth assays

Soft agar assays were performed in six-well plates. Wells were layered with 1.5 ml of 1% agar mixed 1:1 with 2X Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 2% penicillin/streptomycin (pen/strep). A total of 5×10^4 CS-99 Rint+/Gint- cells or a mixture of 2.5×10^4 CS-99 Rint+/Gint- cells and 2.5×10^4 cells from each E-cadDipIIIcI[2] clone were seeded in 0.35% agar in DMEM supplemented with 10% FBS and 1% pen/strep. Colonies were allowed to grow for two weeks. Colonies were imaged using epifluorecence microscopy as described above, and the number of colonies was quantified using ImageJ.

Bioinformatics and pathway analysis

Gene expression data was obtained from parental DU145 cells and cells that underwent serial intravenous injections and metastasis in the lungs (Banyard et al. 2013). This publically-available dataset (GSE51755) was analyzed by GEO2R to compare expression of EMT biomarkers between the first two passages and the last two passages. Genes that were significantly upregulated (p<0.05) in the first two passages and in the last two passages were input as gene lists into FuncAssociate (Berriz et al., 2009) to identify Gene Ontology (GO) terms that were significantly enriched in the upregulated gene lists from passages one and two or passages three and four. Gene expression of EMT markers was compared between primary prostate cancer samples and bone metastases (Stanbrough et al. 2006; 16510604; GSE32269) using GEO2R.

Statistical Analyses

Differences between luciferase outputs from the various reporters (Figure 2) were analyzed using one-way analysis of variance with Tukey's post-hoc correction. QRT-PCR results were tested for significance using a Student's t-test (Figure 3). Percentages of MET from the lineage tracing experiments (Figure 4) and the number of metastases from suicide reporter experiments (Figures 5, 7, and supplementary Figure 1) were analyzed by the Kruskal-Wallis test. Differences in growth rates between CS-99 E-cadDipIIIcI[2] clones and Rint+/Gint- cells and differences in the number of colonies formed in soft agar were analyzed by analysis of variance. All analyses were performed using JMP (version 9.0).
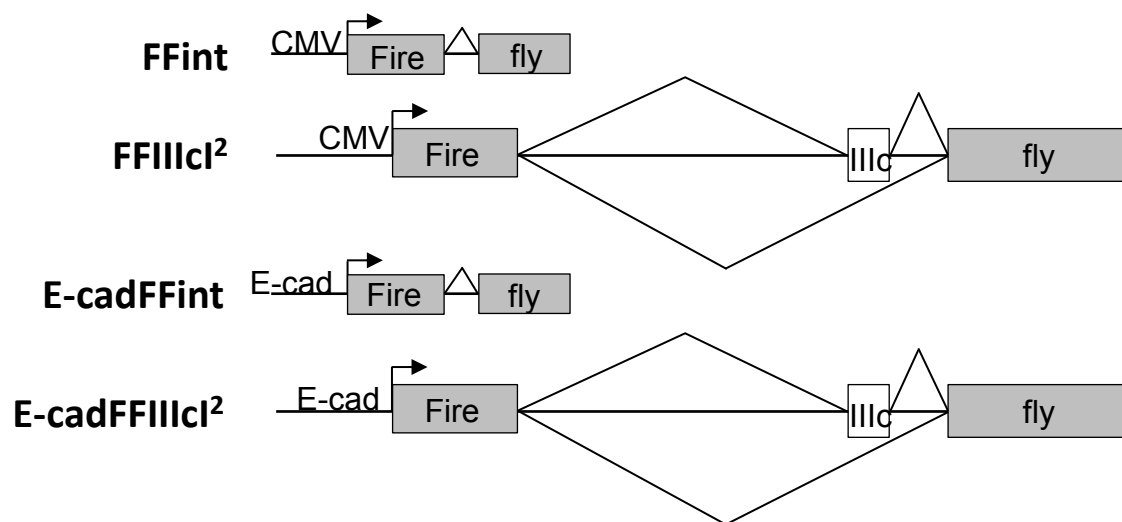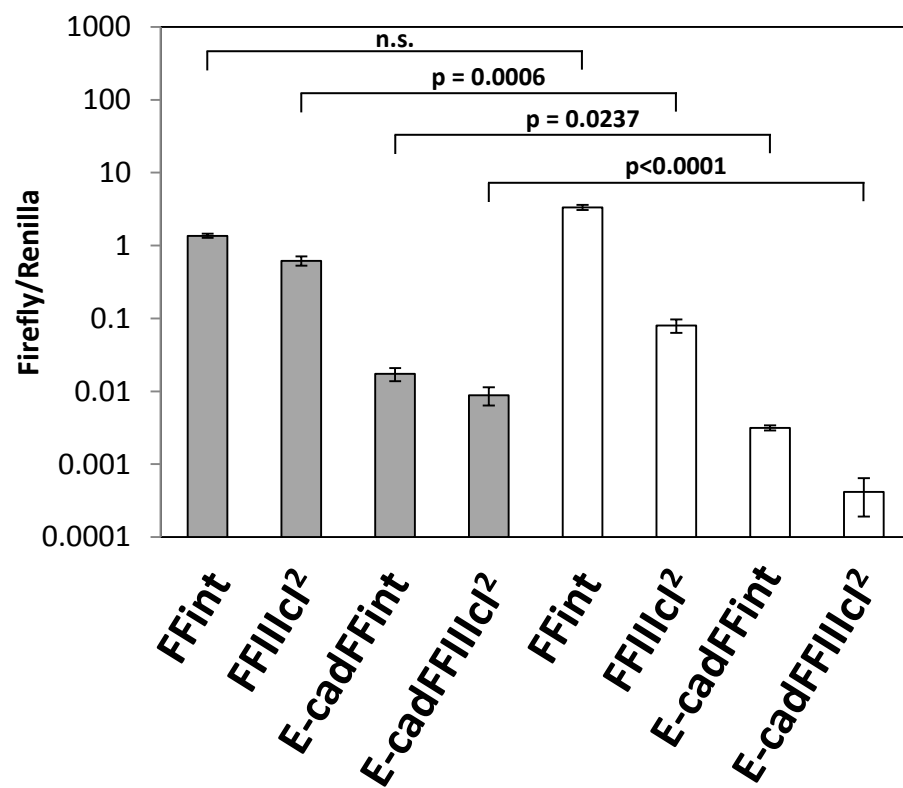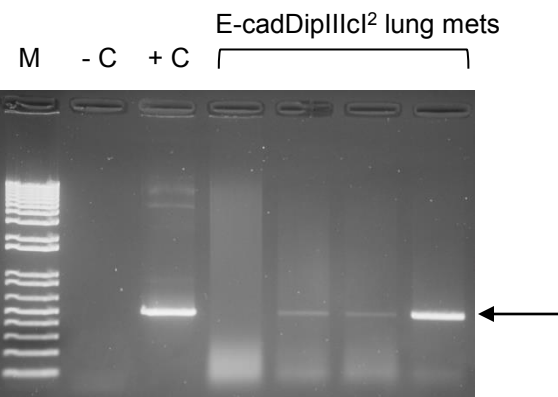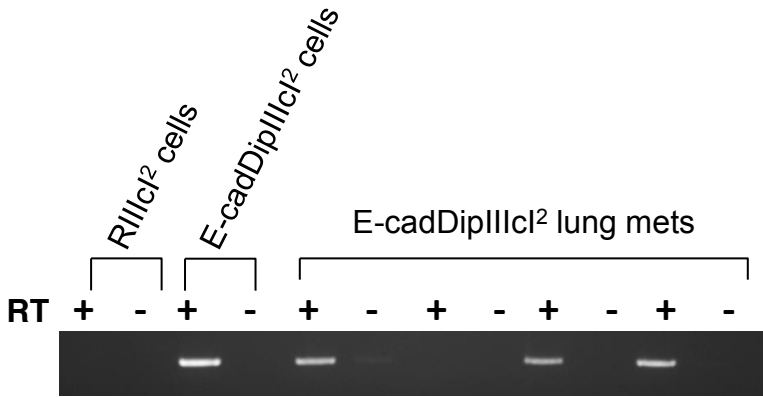
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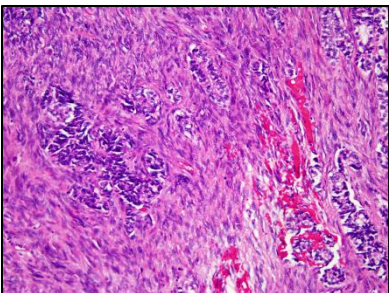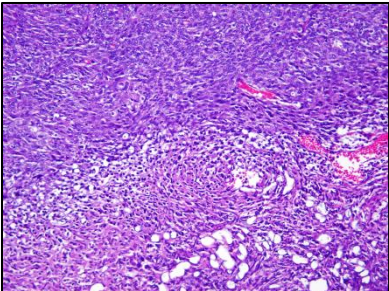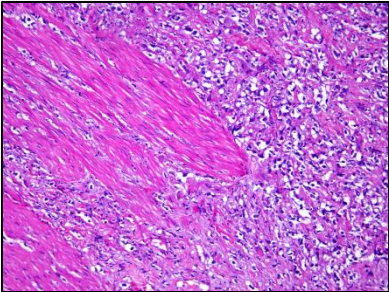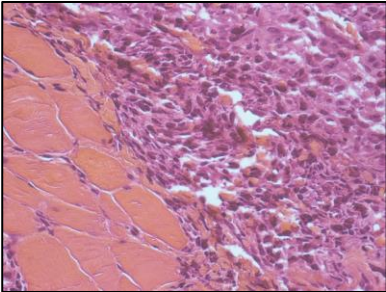**Human carcinosarcomas**

AT3 primary tumor

B

	Cytokeratin	**Vimentin**

CS

AT3